TOXICOLOGICAL SCIENCES **120(2)**, 460–474 (2011)
doi:10.1093/toxsci/kfr020
Advance Access publication January 25, 2011

Species-Specific Dibutyl Phthalate Fetal Testis Endocrine Disruption Correlates with Inhibition of SREBP2-Dependent Gene Expression Pathways

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Received November 29, 2010; accepted January 14, 2011

Fetal rat phthalate exposure produces a spectrum of male reproductive tract malformations downstream of reduced Leydig cell testosterone production, but the molecular mechanism of phthalate perturbation of Leydig cell function is not well understood. By bioinformatically examining fetal testis expression microarray data sets from susceptible (rat) and resistant (mouse) species after dibutyl phthalate (DBP) exposure, we identified decreased expression of several metabolic pathways in both species. However, lipid metabolism pathways transcriptionally regulated by sterol regulatory element–binding protein (SREBP) were inhibited in the rat but induced in the mouse, and this differential species response corresponded with repression of the steroidogenic pathway. In rats exposed to 100 or 500 mg/kg DBP from gestational days (GD) 16 to 20, a correlation was observed between GD20 testis steroidogenic inhibition and reductions of testis cholesterol synthesis endpoints including testis total cholesterol levels, *Srebf2* gene expression, and cholesterol synthesis pathway gene expression. SREBP2 expression was detected in all fetal rat testis cells but was highest in Leydig cells. Quantification of SREBP2 immunostaining showed that 500 mg/kg DBP exposure significantly reduced SREBP2 expression in rat fetal Leydig cells but not in seminiferous cords. By Western analysis, total rat testis SREBP2 levels were not altered by DBP exposure. Together, these data suggest that phthalate-induced inhibition of fetal testis steroidogenesis is closely associated with reduced activity of several lipid metabolism pathways and SREBP2-dependent cholesterologenesis in Leydig cells.

Key Words: **phthalate; developmental toxicity; prenatal; reproductive and developmental toxicology; sterol regulatory element–binding protein; fetal testis; endocrine disruptors; microarray.**

Phthalates are esters of phthalic acid produced in high volume and used in a variety of consumer products including cosmetics, medical devices, building supplies, and food packaging. Because of a noncovalent linkage to the product matrix, phthalates leech from materials during normal use leading to ubiquitous, daily human exposure (Koch and Calafat, 2009; Latini, 2005). In human male infants, hypospadias and undescended testes are prevalent congenital malformations (Foresta *et al.*, 2008), and phthalate exposure is hypothesized to contribute to these malformations (Foster, 2006). Some epidemiological data support this hypothesis (Main *et al.*, 2006; Swan, 2008). Pregnant rats exposed to high levels of certain phthalate congeners during a critical late gestational window produce male pups with a spectrum of reproductive tract malformations such as hypospadias, retained nipples, and undescended testes (Carruthers and Foster, 2005). These malformations result from rapid inhibition of rat Leydig cell testosterone and insulin like-3 production after phthalate exposure (Thompson *et al.*, 2004; Wilson *et al.*, 2004).

The molecular mechanism of phthalate-induced steroidogenic inhibition is largely unknown. Although male reproductive tract malformations have a critical window of susceptibility, phthalates inhibit fetal rat Leydig cell testosterone production at any gestational age of active steroidogenesis (Scott *et al.*, 2008). Inhibition of Leydig cell testosterone synthesis is explained at least in part by reductions in messenger RNA (mRNA) levels of several steroidogenic genes including *Cyp11a1*, *Cyp17a1*, *Scarb1*, and *Star* (Barlow *et al.*, 2003; Lehmann *et al.*, 2004). Phthalates also reduce neutral lipid content in fetal Leydig cells (Barlow *et al.*, 2003; Lehmann *et al.*, 2004). Diminished binding of the transcription factor CCAAT/enhancer binding protein beta to steroidogenic gene promoters may contribute to attenuation of Leydig cell steroidogenic gene expression after phthalate exposure (Kuhl *et al.*, 2007). Phthalates cause aggregation and reduction of volume of Leydig cells (Mahood *et al.*, 2005; Mylchreest *et al.*, 2002), but the total number and proliferation rate of fetal Leydig cells appear unchanged.

In utero phthalate exposure produces species-specific phenotypes. Although some phthalate-induced phenotypes

TABLE 1
DBP Treatment Groups

Microarray and Pathway Analysis Experiments

Species	Dose level mg/kg	Exposure age	Dosing regimen	Necropsy timing after final dose (h)	Group size	Affymetrix microarray	Significant probe sets[a]	Reference
Rat	500	GD19	Single	1, 3, 6, and 18	3–4	RAE230A + B[b]	2841	Thompson *et al.* (2004)
Rat	500	GD12–GD19	Daily	Varied: 2–8	3	RAE230A + B	389	Liu *et al.* (2005)
Rat	50	GD12–GD20	Daily	6	4	Rat230v2	457	This article
Mouse	500	GD18	Single	2, 4, and 8	3	Mouse430v2	3473	Gaido *et al.* (2007)
Mouse	250	GD14–GD17	Daily	2	3	Mouse430v2	510	Gaido *et al.* (2007)

SREBP expression and function experiments

Species	Dose level mg/kg	Exposure age	Dosing regimen	Necropsy timing after final dose (h)	Group size
Rat	100	GD12–GD20	Daily	6	5
Rat	500	GD12–GD20	Daily	6	5

[a]Values are the number of probe sets in the entire study with an FDR q value < 0.05.
[b]The RAE230A chip was employed at all time points, but the RAE230B chip was not used at 6 and 18 h time points.

such as gonocyte multinucleation are similar between mice and rats, mice are refractory to inhibition of both fetal testosterone production and expression of fetal testis steroidogenic genes (Gaido *et al.*, 2007). Thus, some phthalate-induced phenotypes are attributed to reduced Leydig cell hormone production, whereas others, such as gonocyte multinucleation, utilize a different mechanism. In the gut, orally ingested phthalate diester is quantitatively metabolized to its proximate toxicant, phthalate monoester. However, there appears to be no significant difference in phthalate diester metabolism or placental transfer of the proximate toxicant to the fetus between rats and mice (Gaido *et al.*, 2007). *In utero* phthalate exposure of marmosets perturbs normal germ cell development but does not reduce masculinization, suggesting that the phthalate effect on primates is similar to its effect on mice (McKinnell *et al.*, 2009).

Sterol regulatory element–binding protein (SREBP) transcription factors are master regulators of fatty acid and cholesterol synthesis (Brown and Goldstein, 2009; Osborne and Espenshade, 2009). Precursor SREBP reside in the endoplasmic reticulum, but in response to low cholesterol or fatty acid levels, these proteins translocate to the Golgi complex where proteases generate small active fragments that migrate into the nucleus and function as transcription factors. Mammals express two SREBP homologs, SREBP1 and SREBP2. Differential promoter usage generates two SREBP1 isoforms (SREBP1a and SREBP1c) from the *Srebf1* gene (Hua *et al.*, 1995). In general, it is thought that SREBP1c primarily regulates expression of fatty acid synthesis pathway genes, SREBP2 primarily mediates expression of cholesterol synthesis pathway genes, and SREBP1a activates expression of genes in both pathways. Proteins in the cholesterol synthesis pathway are highly expressed in fetal mouse Leydig cells (Budefeld *et al.*, 2009). Interestingly, *Star* and *Cyp17a1* expression in cultured cells is enhanced by SREBP transcriptional activity (Christenson *et al.*, 2001; Ozbay *et al.*, 2006; Shea-Eaton *et al.*, 2001). It is not yet known whether SREBP perform these steroidogenic functions in the fetal testis.

Species-specific endocrine disruption effects highlight the need to understand the phthalate molecular mechanism to determine if phthalate exposure could contribute to human reproductive malformations. In this study, we exploited species-specific effects of phthalates by bioinformatically mining mRNA expression profiling data to identify molecular pathways that correlate with dibutyl phthalate (DBP)-induced endocrine disruption. This analysis gave rise to a hypothesis that reduced fetal Leydig cell testosterone production after exposure is associated with attenuation of SREBP expression and activity.

MATERIALS AND METHODS

Data from several phthalate exposure studies were examined (Table 1). For the bioinformatic pathway analysis, primary data (CEL) files were obtained from Dr Kevin Gaido (U.S. Food and Drug Administration) from four previously published phthalate exposure experiments of Sprague-Dawley rats or CD1 mice (Gaido *et al.*, 2007; Liu *et al.*, 2005; Thompson *et al.*, 2004). Animal usage, phthalate exposure paradigms, and microarray data generation for these published experiments are not described in detail here. Also included in the bioinformatic pathway analysis was an additional expression microarray experiment performed following a 50 mg/kg DBP (DBP50) exposure of Fischer 344 rats from gestational day (GD) 12 to 20. For the experiments examining the affects of DBP on SREBP, DBP100 and DBP500 Sprague-Dawley rat exposures were from GD16 to GD20, and each DBP group had a distinct

vehicle control group. Animal usage, exposure paradigms, and molecular techniques for both the DBP50 microarray experiment and the SREBP experiments are detailed below.

Animals and phthalate exposure. For the DBP50 microarray experiment, timed-pregnant Fischer 344 rats were obtained from Charles River Laboratories (Raleigh, NC), housed in The Hamner Institutes (Durham, NC) vivarium in polycarbonate cages containing cellulose bedding, fed NIH-07 rodent chow (Zeigler Brothers, Gardener, PA), and provided reverse-osmosis water *ad libitum*. For the studies analyzing SREBP biology, timed-pregnant Sprague-Dawley rats were purchased from Charles River Laboratories, housed in the Alfred I. duPont Hospital for Children vivarium in polycarbonate cages containing pine shavings, fed LabDiet Rat Chow 5012 (PMI Nutrition International, Brentwood, MO), and provided with tap water *ad libitum*. Both vivariums are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all animal protocols were approved by the Institutional Animal Care and Use Committee. The day of sperm-positive identification was designated GD0.

For DBP (CAS number 84-74-2; Sigma Chemical Co., St Louis, MO) exposures, groups of weight-randomized rats were gavaged each day of the study between 8:00 and 10:00 A.M. with 1 ml/kg (body weight) of a DBP solution in corn oil (Sigma Chemical Co.) or corn oil alone. DBP dose levels between 50 and 500 mg/kg body weight were chosen to produce no significant effect on fetal rat Leydig cell steroidogenesis (DBP50) or to produce profound inhibition (DBP500). For comparison, average human phthalate exposure is in the range of micrograms per kilogram per day, although certain subpopulations may be exposed to low milligrams per kilogram per day amounts (Wittassek *et al*., 2010). On the day of tissue procurement, animals were euthanized 6 h after the final DBP dosing. Prior to euthanasia, pup anogenital distance was measured with a stereomicroscope fitted with a 1-mm reticle. Testes were placed in microcentrifuge tubes and immediately frozen on dry ice for subsequent testosterone, mRNA, or protein analyses.

Testis histology. Paraffin sections were stained with hematoxylin and eosin, and multinucleated germ cell (MNG) formation and seminiferous cord diameters were quantified as previously described (Johnson *et al*., 2008).

Fetal testis cholesterol and testosterone measurements. Testis total cholesterol was measured from two pooled testes (each from different fetuses) per litter using a colorimetric cholesterol quantitation kit (K603-100; Biovision Inc., Mountain View, CA) according to the manufacturer's protocol.

When possible, two testes (each from different fetuses) per litter were pooled for testosterone analysis. For the DBP50 expression microarray experiment, a radioimmunoassay was performed as previously described using a Double-Antibody-125I RIA kit (Catalog #07-189105; MP Biomedicals, Costa Mesa, CA) (Johnson *et al*., 2007). For the SREBP study, testes were homogenized in 100 µl PBS, centrifuged at 15,000 × g for 5 min, and testosterone measured in the supernatant by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Lab using a radioimmunoassay kit (Catalog #TKTT2; Siemens Medical Solutions Diagnostics, Los Angeles, CA).

DBP50 microarray. For the DBP50 exposure microarray, fetal testes were extracted with TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and total RNA purified using an RNeasy Micro kit (Qiagen, Valencia, CA). RNA quality was measured with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and all RNA samples showed an RNA Integrity Number greater than 8. Biotin-labeled complementary RNA probes were generated using the One Cycle Target Labeling and Control Reagents kit (catalog number 900943; Affymetrix, Santa Clara, CA). Probe hybridization to Affymetrix Rat Genome 230 2.0 microarrays and microarray washing and scanning were performed as previously described (Lahousse *et al*., 2006). The DBP50 microarray data have been submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and given accession number GSE25196.

Microarray statistical analysis. Data were analyzed statistically from five fetal testis expression Affymetrix microarray experiments after DBP exposure (Table 1). Although different normalization and statistical methods were used to analyze the microarray data described in previous publications, all primary microarray data were subjected to uniform data normalization and statistical analysis in this study. Data were normalized using the Robust MultiChip Average method (Irizarry *et al*., 2003), and raw values were log transformed. Using Array Studio software (OmicSoft Corp., Morrisville, NC), one-way ANOVA was used to compare overall expression across microarray data in an experiment and was followed by Student's *t*-tests to compare the expression levels of group pairs of interest. A false discovery rate (FDR) q value < 0.05 was considered significant. Calculated fold change and FDR q values for all microarray data sets are displayed in Supplementary files 1 and 2.

Bioinformatic pathway analysis. Two complementary methods were used to identify biological pathways significantly altered by phthalate exposure. Both methods determine if genes within a biological pathway are significantly enriched (i.e., overrepresented) in gene lists identified as being altered by experimental treatment; however, the lists of genes used as the input data and the statistical analysis of the two methods are different. The first method, performed with Ingenuity Pathways Analysis (IPA) version 8.7 software (Ingenuity Systems, www.ingenuity.com), used lists of genes having statistically significant differential expression as input data and a right-tailed Fisher exact test, whereas the gene set enrichment analysis (GSEA) method used expression fold change data from all genes examined as the input data and a Kolmogorov-Smirnov running sum statistic (Subramanian *et al*., 2005). For IPA analysis, the pathways queried for overrepresentation included canonical metabolic pathways, which were derived from Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) annotations. For GSEA, lipid, carbohydrate, amino acid, nucleotide, and energy metabolism pathways curated by KEGG were examined. In addition, custom gene sets were queried using IPA and GSEA. These custom gene sets included the testicular steroidogenic, fatty acid biosynthesis, and cholesterol biosynthesis pathways and gene sets whose expression was influenced by SREBP1 or SREBP2 transcriptional activity, as determined using the IPA Knowledge Base (Supplementary file 3). The IPA parameters employed were the following: (1) genes were imported in files using gene symbols as the identifier; (2) the reference gene background was set to the genes present on the Affymetrix microarray used in the experiment; and (3) a Benjamini-Hochberg q value < 0.05 was considered significant. GSEA was performed using the online implementation tool (version 2.0) provided by the Broad Institute (www.broadinstitute.org/gsea/index.jsp). GSEA parameters were the following: (1) the imported data set was collapsed to gene symbols, (2) 1000 permutations were performed using gene set as the permutation type, (3) gene sets containing 10–500 genes were analyzed, and (4) FDR q values < 0.05 were considered significant.

Quantitative Reverse Transcription-Polymerase Chain Reaction. Total RNA purification, complementary DNA generation, Taqman-based PCR, and data analysis using the delta-delta threshold cycle method were performed as described (Barthold *et al*., 2008), except for performing technical duplicate amplifications. Because DBP exposure at 500 mg/kg significantly reduced fetal testis *Gapdh* expression (data not shown), target gene expression was determined relative to *Tbp* expression. Fetal testis *Tbp* mRNA levels were not affected by DBP exposure (data not shown). The following prevalidated Taqman assays (Applied Biosystems Inc., Foster City, CA) were used—*Cyp11a1*: Rn00568733_m1, *Cyp17a1*: Rn00562601_m1, *Hmgcr*: Rn00565598_m1, *Idi1*: Rn00585526_m1, *Insig1*: Rn00574380_m1, *Scarb1*: Rn00580588_m1, *Srebf2*: Rn01502638_m1, *Srebf1c*: Rn01495766_m1, *Star*: Rn00580695_m1, and *Tbp*: Rn01455646_m1. To specifically quantify *Srebf1a* expression, no premade Taqman assay was available; therefore, a custom assay spanning the first two exons of *Srebf1a* was designed and validated by Applied Biosystems. For the *Srebf1a* Taqman assay, PCR primer sequences were 5′ACGACGGAGCCATGGATTG and 5′AGTCACTGTCTTGGTTGTTGATGAG and the Taqman probe sequence was 5′AAGCATGTCTTCAAATGTG

Immunostaining and quantification of SREBP2 immunoexpression. An immunofluorescent technique using paraffin sections was employed. Following dissection, fetal testes were immersed in Bouin's fixative for 1 h, embedded in paraffin, and antigen retrieval performed on 5-µm deparaffinized sections. For antigen retrieval, slides were washed for 10 min with PBS

TABLE 2
IPA Canonical Pathway Enrichment Analysis of Mouse and Rat Testis Expression Microarrays

	Fetal rat exposure						Fetal mouse exposure			
	DBP50	DBP500					DBP250	DBP500		
IPA canonical pathway	GD12–20	1 h GD19	3 h GD19	6 h GD19	18 h GD19	GD12–19	GD14–17	2 h GD18	4 h GD18	8 h GD18
Lipid metabolism										
Fatty acid elongation in mitochondria				0.003						
Biosynthesis of steroids					0.0006	<0.0001				
Fatty acid metabolism					0.01	0.005				
Carbohydrate metabolism										
Propanoate metabolism				0.008	<0.0001	0.0002				
Inositol metabolism				0.04	0.002	0.0001				
Galactose metabolism				<0.05		0.02				
Pentose phosphate pathway				0.04		0.0006				
Butanoate metabolism					<0.0001	<0.0001				
Glycolysis/gluconeogenesis					0.0008	<0.0001				
Citrate cycle					0.04	0.0006				
Pyruvate metabolism					0.04	0.0005				
Fructose and mannose metabolism						0.001				
Amino acid metabolism										
Valine, leucine, isoleucine degradation				0.0005	0.001	0.0003				
Glycine, serine, threonine metabolism				<0.05						
Phenylalanine metabolism					0.005	0.0001				
Tryptophan metabolism					0.03	0.004				
Histidine metabolism					0.04	0.006				
Glutathione metabolism						0.0001				
Tyrosine metabolism						0.02				
Lysine degradation						0.02				
Nucleotide metabolism										
Pyrimidine metabolism									0.004	0.004
Purine metabolism										0.005
Energy metabolism										
Oxidative phosphorylation						0.02				0.005

Note. Numerical values in the table are Benjamini-Hochberg q values. In cells without values, IPA canonical pathway enrichment analysis had q values > 0.05. At each GD and DBP exposure, shaded values indicate that most or all of genes within the functional category showed decreased expression.

(pH 7.4) with 0.05% Tween-20 (PBST) and immersed for 40 min in 85°C citrate buffer (10 mM trisodium citrate, 0.05% Tween-20, pH 6.0). After washing twice with PBST containing 0.1% Triton X-100, tissue sections were blocked for 30 min with PBST containing 10% normal goat serum (blocking buffer). Sections were incubated overnight at 4°C with primary antibodies diluted in blocking buffer, washed three times with PBST, and incubated for 1 h at room temperature in fluorophore-conjugated secondary antibody diluted in PBST. After washing three times with PBST, Vectashield mounting media (Vector Labs, Burlingame, CA) was used to mount a glass coverslip. Primary antibodies and working concentrations were 4 μg/ml rabbit anti-SREBP2 (ab30682; Abcam, Cambridge, MA), rabbit anti-CYP11A1 (AB1294; Millipore Corp., Billerica, MA) diluted 1:500, mouse anti-smooth muscle alpha actin (ACTA2) (clone 1A4; Sigma Chemical Co.) diluted 1:200, and 2.5 μg/ml normal rabbit immunoglobulin G (IgG) (I8140; Sigma Chemical Co.). Alexa594-conjugated goat anti-rabbit IgG (Invitrogen Corp.) and Alexa488-conjugated goat anti-mouse IgG (Invitrogen) secondary antibodies were diluted 1:500. Using a ×10 UPlanFl objective mounted on an Olympus BX60 epifluorescence microscope and Image-Pro plus software (Media Cybernetics Inc., Bethesda, MD), images encompassing portions of the testis section were captured and assembled into a single image using the Photoshop CS3 (Adobe Systems Inc., San Jose, CA) photomerge function.

For SREBP2 quantification, all tissue section images were captured using identical settings, and immunostaining intensity in different tissue compartments was measured with ImageJ software (http://rsb.info.nih.gov/ij/). Using the freehand selection tool, regions of interest encompassing the entire testis section, individual Leydig cell clusters, or individual seminiferous cords were selected, and the mean pixel intensity within each region of interest was obtained. From one fetus per litter, approximately 30 Leydig cell clusters or seminiferous cords were quantified. For fetuses exposed to DBP500, however, Leydig cell aggregation reduced the numbers of Leydig cell clusters measured to 5–10 per litter. Mean pixel intensity values for individual Leydig cell clusters or seminiferous cords were averaged to obtain a single value for each litter.

Western blotting. Standard techniques were employed. Fetal testes were homogenized in buffer (150 mM NaCl, 1% sodium deoxycholate, 0.1%

TABLE 3
GSEA of Fetal Mouse and Rat Testis Gene Expression Microarrays

	Fetal rat exposure						Fetal mouse exposure			
	DBP50	DBP500					DBP250	DBP500		
KEGG gene set	GD12–20	1 h GD19	3 h GD19	6 h GD19	18 h GD19	GD12–19	GD14–17	2 h GD18	4 h GD18	8 h GD18
Lipid metabolism										
Biosynthesis of steroids				<0.0001	<0.0001	<0.0001	<0.0001			
Fatty acid metabolism				0.004	0.001	<0.0001			0.02	
Polyunsaturated fatty acid biosynthesis				0.006	0.0002	0.004			0.04	0.03
Fatty acid elongation in mitochondria				0.02	0.04	0.02				
Alpha linolenic acid metabolism				0.02						
Arachidonic acid metabolism				0.04						
Bile acid biosynthesis						0.006				
Carbohydrate metabolism										
Propanoate metabolism				0.001	<0.0001	<0.0001			0.009	0.02
Citrate cycle				0.006	0.001	0.001				
Pyruvate metabolism				0.007	0.02	<0.0001			0.04	
Glyoxylate and dicarboxylate metabolism					0.006	0.009				
Butanoate metabolism					0.006	<0.0001			0.03	
Glycolysis and gluconeogenesis					0.02	0.0008			0.01	
Pentose phosphate pathway						0.03				
Amino acid metabolism										
Tryptophan metabolism				0.0005	0.004	<0.0001			0.01	0.01
Lysine degradation				0.006	0.002	<0.0001			0.02	
Glutathione metabolism					0.01	0.001				<0.0001
Histidine metabolism					0.04	0.006			0.02	0.008
Beta alanine metabolism						0.0005				
Glutathione metabolism										
Phenylalanine metabolism						<0.05				
Arginine and proline metabolism									0.02	
Alanine and aspartate metabolism									0.03	0.01
Selenoamino acid metabolism									0.03	0.01
Glycine serine and threonine metabolism										0.01
Nucleotide metabolism										
Pyrimidine metabolism									0.009	0.005
Purine metabolism									0.04	0.03
Energy metabolism										
Oxidative phosphorylation	0.004			0.007	0.0002	0.03			0.01	0.002

Note. Numerical values in the table are FDR q values. In cells without values, gene sets had GSEA q values > 0.05. At each GD and DBP exposure, shaded values indicate decreased gene set expression and nonshaded values indicate increased gene set expression.

sodium dodecyl sulfate, 5 mM EDTA, and 50 mM Tris, pH 7.4) containing Complete Protease Inhibitor Cocktail (04693132001; Roche Applied Science, Indianapolis, IN), centrifuged at 15,000 × g for 10 min, and supernatant protein concentration determined using a bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL). Ten micrograms protein per sample was separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After incubating for 1 h at room temperature in PBS containing 5% nonfat dry milk and 0.01% Tween-20 (blocking buffer), membranes were exposed to primary antibodies diluted in blocking buffer overnight at 4°C, washed, and exposed to horseradish peroxidase–conjugated secondary antibodies diluted in blocking buffer for 1 h at room temperature. Secondary antibodies were detected using enhanced chemiluminescence, and signal was captured using a Geliance 600 imaging system (Perkin Elmer, Shelton, CT). Primary antibodies and working dilutions were the following: 4 μg/ml rabbit anti-SREBP2 (ab30682; Abcam); mouse beta actin (ACTB) antibody diluted 1:25,000 (clone AC-15; Sigma Chemical Co.); and 2.5 μg/ml normal rabbit IgG (I8140; Sigma Chemical Co.). Horseradish peroxidase–conjugated secondary antibodies and working dilutions were sheep anti-mouse IgG and donkey anti-rabbit IgG diluted 1:2500 (RPN2124; GE Healthcare, Piscataway, NJ). Using ImageJ software, SREBP2 levels were quantified relative to ACTB expression.

Nonmicroarray statistical analysis. Statistical significance of all nonmicroarray data was determined with Graphpad Prism 5.0 software (Graphpad Software Inc., La Jolla, CA) using a two-tailed unpaired t-test. A p value < 0.05 was considered significant.

TABLE 4
IPA and GSEA Analysis of Cholesterol Synthesis, Steroid Synthesis, and SREBP Gene Sets

	Fetal rat exposure						Fetal mouse exposure			
	DBP50	DBP500					DBP250	DBP500		
IPA	GD12–20	1 h GD19	3 h GD19	6 h GD19	18 h GD19	GD12–20	GD14–17	2 h GD18	4 h GD18	8 h GD18
Steroid synthesis				0.0002	<0.0001	<0.0001				
Cholesterol synthesis				0.007	<0.0001	<0.0001	<0.0001			
Fatty acid synthesis				0.004	0.004	<0.0001				
Downstream of SREBP2				0.0002	<0.0001	<0.0001	<0.0001			
Lipid metabolism genes targeted by SREBP1				0.007	<0.0001	<0.0001	0.0005			
GSEA										
Steroid synthesis				<0.0001	<0.0001	<0.0001	0.006			
Cholesterol synthesis			0.003	<0.0001	<0.0001	<0.0001	<0.0001			
Fatty acid synthesis				0.003	0.0002	0.0009	0.02			
Downstream of SREBP2				<0.0001	<0.0001	<0.0001	<0.0001			
Lipid metabolism genes targeted by SREBP1				<0.0001	<0.0001	<0.0001	<0.0001			

Note. Numerical values in the table are Benjamini-Hochberg q values and FDR q values of the IPA and GSEA pathway analysis, respectively. In cells without values, gene set enrichment had q values > 0.05. At each GD and DBP exposure, shaded values indicate that most or all genes within the gene set showed decreased expression and nonshaded values indicate the gene set showed increased expression.

RESULTS

To generate a rat fetal testis expression microarray data set at a DBP dose level approximating a no observed effect level for steroidogenic inhibition (Lehmann *et al.*, 2004), rats were exposed to corn oil or DBP50 from GD12 to 20, and testis gene expression was queried at GD20 using Affymetrix whole-genome microarrays. DBP50 exposure did not affect maternal or pup body weights (data not shown), and GD20 intratesticular testosterone levels and male anogenital distance were similar (Supplementary file 4). However, a significant increase in multinucleated germ cells was observed. After applying an FDR multiple testing correction, DBP50 exposure significantly altered the fetal testis expression of 457 probe sets (Supplementary file 1). However, testosterone synthesis pathway genes did not show significant changes in expression (Supplementary file 1).

Pathway Analysis of Fetal Testis Expression Microarray Data

Fetal testis expression microarray primary data after DBP exposure of susceptible (rat) and resistant (mouse) species (Table 1) were mined to identify biological pathways correlating with repression of fetal testis steroidogenesis. All raw microarray data were subjected to identical normalization and statistical procedures, and two complementary bioinformatic pathway analysis techniques (IPA and GSEA) were used. In both mouse and rat fetal testes, categories of genes functioning in amino acid, energy, carbohydrate, and lipid metabolism pathways were significantly changed (Tables 2 and 3). Supplementary files 5, 6, and 7 contain the genes present in each significant pathway shown in Tables 2 and 3 and the DBP-responsive genes contributing to significant pathway enrichment. In rats exposed to a DBP dose level (DBP500) causing steroidogenic inhibition, significant enrichment of several metabolic pathways was observed, and gene expression changes within these pathways were nearly always reduced. At the DBP50 dose level that did not reduce rat fetal testis steroidogenesis, the vast majority of metabolic pathways were not altered. Comparing IPA and GSEA techniques revealed that GSEA was the more sensitive method to detect significant metabolic pathway enrichment. Using GSEA, amino acid, energy, and carbohydrate metabolism pathways were identified as significantly changed at earlier time points of rat DBP500 exposure than using IPA, and only GSEA detected significant changes in these pathways at certain time points in the fetal mouse testis.

An informative outcome of GSEA using KEGG-curated pathways was a fetal testis species-specific change in the pathway "biosynthesis of steroids" that correlated with susceptibility to phthalate-induced reductions of testosterone levels (Table 3). The biosynthesis of steroids pathway name is somewhat misleading because most genes included in this pathway are involved in cholesterol biosynthesis from acetyl-CoA, and genes that function in steroid synthesis from cholesterol are not included. Using GSEA, this pathway displayed enrichment beginning 6 h after rat DBP500 exposure, but no enrichment was observed following acute DBP500 exposure in the mouse. Multiple day, DBP250 exposure of the mouse generated significant enrichment in the biosynthesis of steroids pathway; however, genes were decreased in the rat but increased in the mouse. Overall, these data indicated

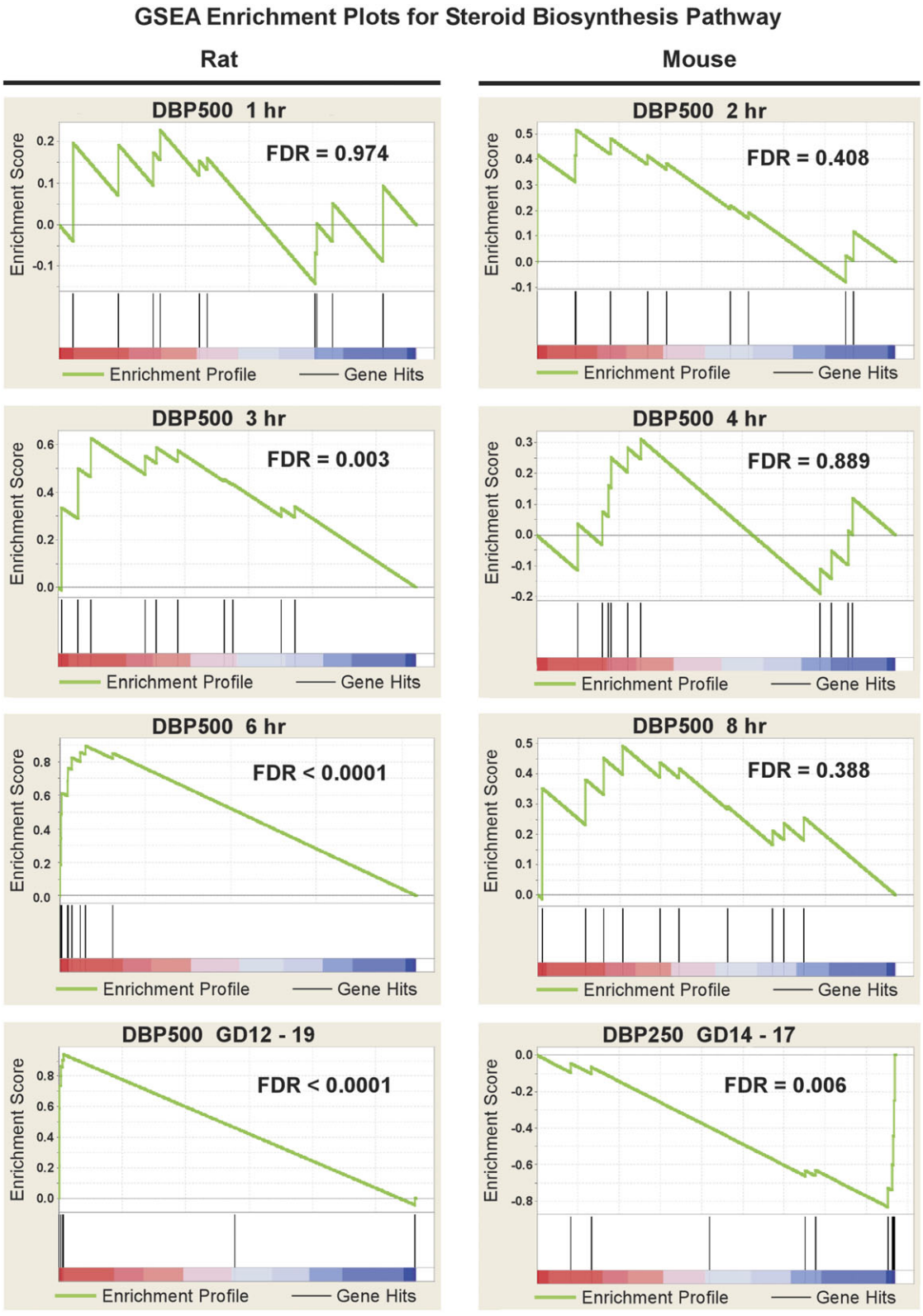

FIG. 1. Steroid biosynthesis pathway GSEA plots for selected DBP exposure paradigms. Data for rat and mouse exposures are shown in the left and right columns, respectively. At the bottom of each plot, the spectrum of gene expression observed on the microarray after DBP exposure is shown, with the left side of the figures indicating reduced expression and the right side of the figures indicating increased expression. The expression of each gene within the pathway is indicated by a ''gene hit'' above the gene expression spectrum.

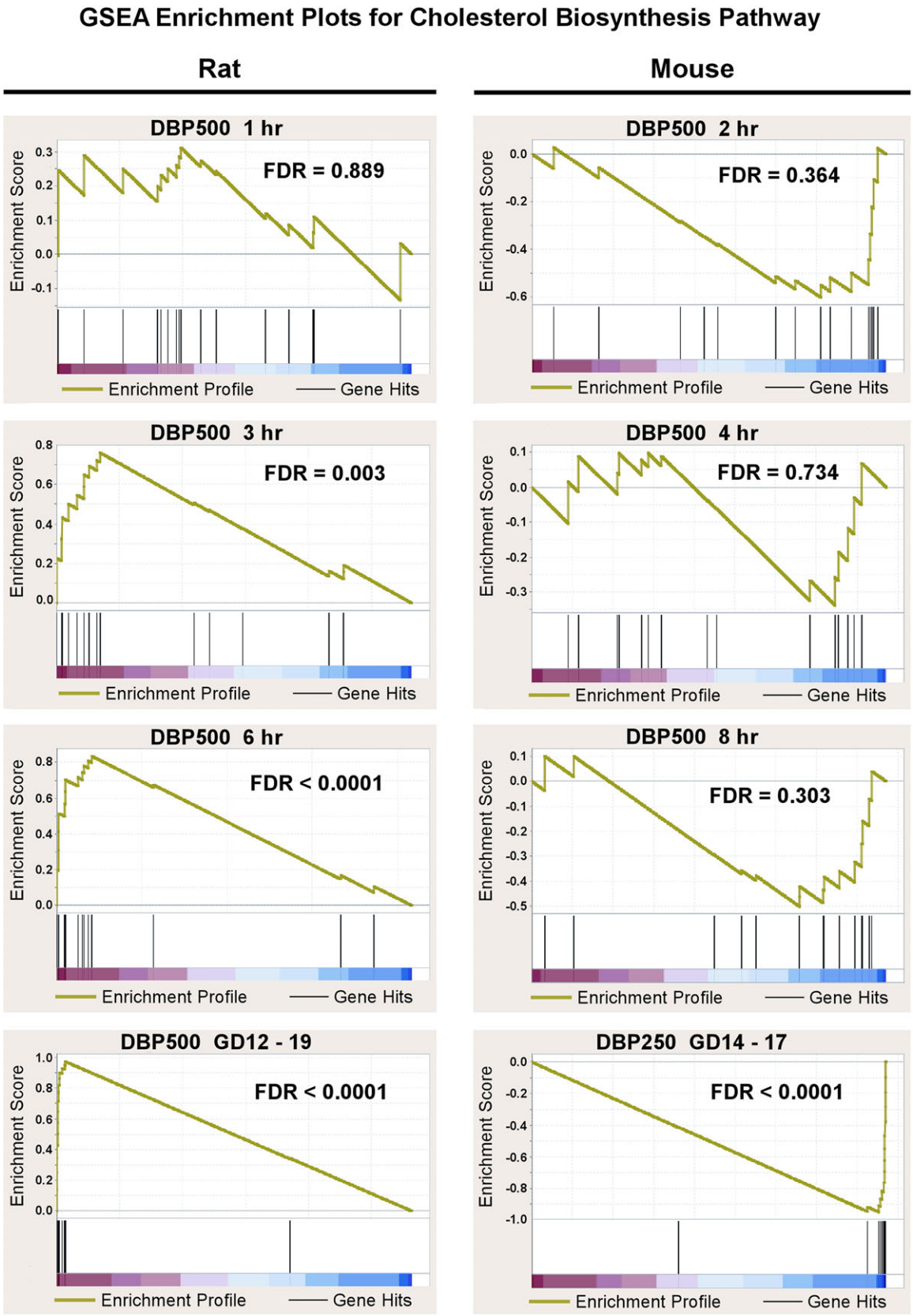

FIG. 2. Cholesterol biosynthesis pathway GSEA plots for selected DBP exposure paradigms. Data for rat and mouse exposures are shown in the left and right columns, respectively. At the bottom of each plot, the spectrum of gene expression observed on the microarray after DBP exposure is shown, with left side of the figure indicating reduced expression and right side of the figure indicating increased expression. The expression of each gene within the pathway is indicated by a ''gene hit'' above the gene expression spectrum.

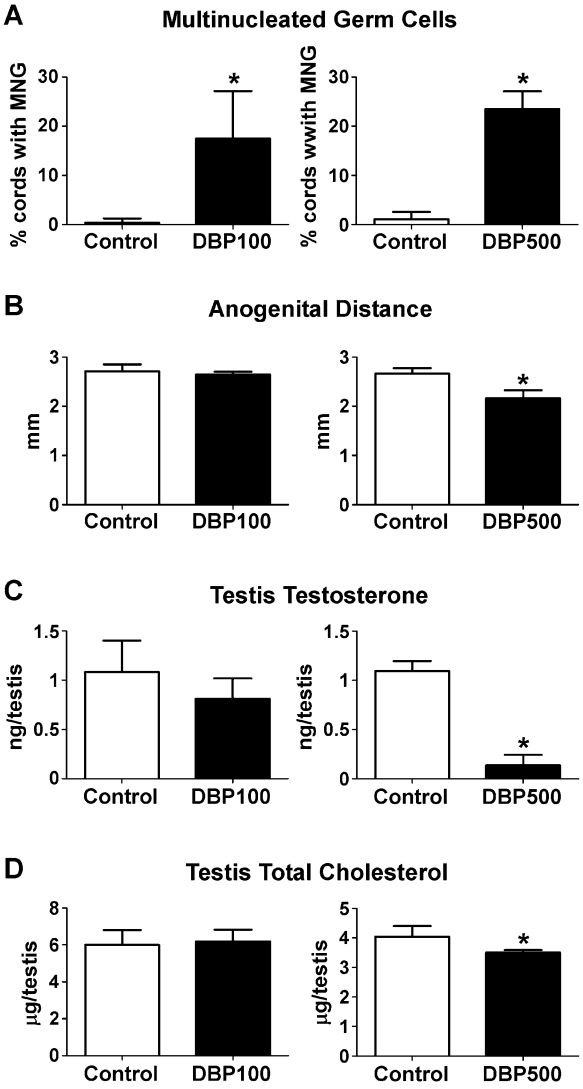

FIG. 3. GD20 testis phenotypes after rat GD12–20 DBP exposure. (A) Percentage of seminiferous cords with one or more multinucleated germ cells. (B) Litter average of male pup anogenital distance. (C) Testis testosterone levels. (D) Testis total cholesterol levels. Group sizes were six control and five DBP-treated litters. All data are the mean ± SD, and an asterisk above a graph indicates a p value < 0.05 compared with control.

that reduction of cholesterologenic gene expression is closely correlated with phthalate-induced endocrine disruption.

In all mammalian tissues, genes in the cholesterol synthesis pathway are regulated by SREBP transcriptional activity. Because of this, we sought to determine if DBP exposure significantly altered fetal testis expression of genes downstream of SREBP1 and/or SREBP2 activity. To generate these custom gene sets, we mined the IPA Knowledge Base for genes regulated by SREBP1 or SREBP2 and produced two gene sets containing 54 and 77 genes (Supplementary file 3). As expected, the vast majority of these SREBP-regulated genes are involved in lipid metabolism, and there was extensive content overlap between the SREBP1 and SREBP2 gene sets. Because no KEGG-curated pathway contains genes specific to fatty acid biosynthesis, cholesterologenesis, and testicular steroidogenesis, we also generated custom gene sets representing these pathways (Supplementary file 3).

After DBP exposure, all five custom gene sets showed similar enrichment profiles (Table 4). All were significantly decreased by rat DBP500 exposure, not altered by acute mouse DBP500 exposure, and significantly increased by multiple day mouse DBP250 exposure. The earliest significant pathway enrichment observed in the rat was a change at 3 h after DBP500 exposure in the cholesterol synthesis pathway. GSEA plots comparing mouse and rat fetal testis expression of steroidogenic (Fig. 1) and cholesterologenic (Fig. 2) gene sets and heat maps derived from GSEA (Supplementary file 8) revealed species-dependent mirror images: several steroidogenic genes including *Star*, *Cyp11a1*, and *Cyp17a1* and cholesterologenic genes including *Hmgcr* and *Idi1* were among the genes with the greatest decreased expression change in rat but the greatest increased expression change in the mouse. From these bioinformatic data, we concluded that DBP-induced reductions in fetal testis testosterone production are closely associated with decreased expression of genes regulated by SREBP, especially those in the cholesterol biosynthesis pathway.

Rat DBP Exposure Inhibits Fetal Testis SREBP2 Expression and Associated Functions

To assess SREBP expression and functions associated with SREBP after rat DBP exposure, we employed an oral gavage model consisting of daily dam exposure to vehicle, DBP100, or DBP500 from GD12 to 20 with endpoint analyses on GD20. In these exposure models, DBP did not affect maternal or pup body weights (data not shown), but the number of multinucleated germ cells was increased significantly after both DBP100 and DBP500 exposures (Fig. 3A). Male anogenital distance was not changed by DBP100 exposure but was reduced by DBP500 (Fig. 3B). DBP500 significantly decreased intratesticular testosterone levels by nearly 90% (Fig. 3C). DBP100 reduced testosterone levels by approximately 20%, but this effect was not statistically significant ($p = 0.14$). Because cholesterol synthesis pathway gene expression was reduced by DBP exposure, intratesticular cholesterol levels were also assayed. Similar to the testosterone data, testis total cholesterol levels were not affected by DBP100 exposure but were significantly reduced by DBP500 (Fig. 3D).

Using quantitative RTPCR, we examined the fetal testis mRNA levels of *Srebf1a*, *Srebf1c*, and *Srebf2* (encoding SREBP1a, SREBP1c, and SREBP2, respectively) and a subset of cholesterol metabolism genes transcriptionally increased by SREBP activity (Fig. 4). These data were correlated with the fetal testis mRNA expression pattern of steroidogenic genes known to be decreased by rat DBP exposure. In agreement with the intratesticular testosterone dose-response, DBP500 (but not DBP100) significantly reduced expression of *Cyp11a1*, *Cyp17a1*, *Scarb1*, and *Star*. *Srebf1a* and *Srebf1c* expression

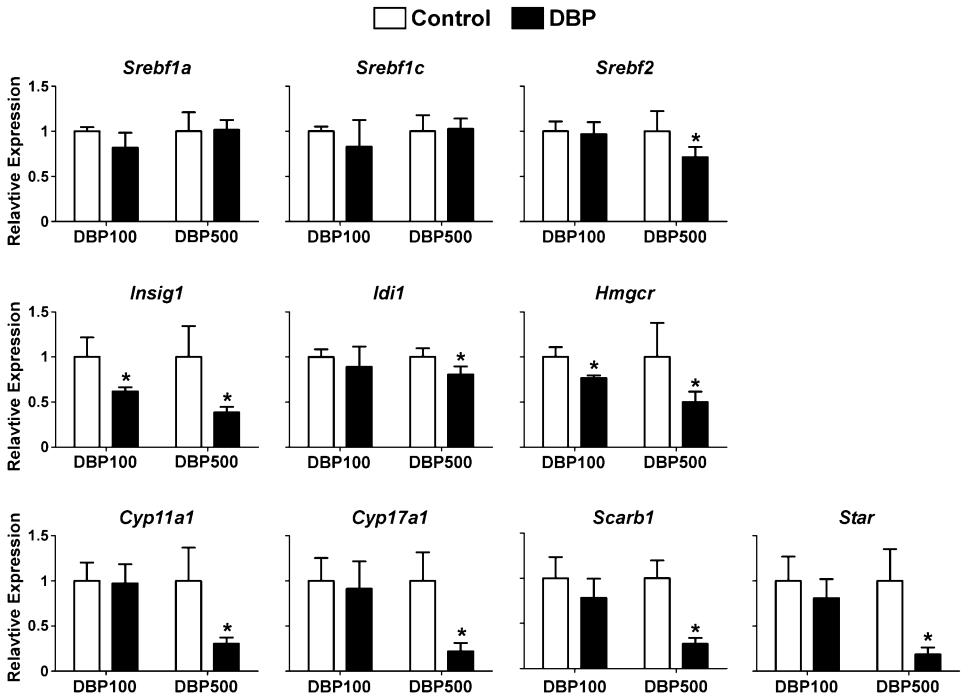

FIG. 4. GD20 rat testis cholesterologenic and steroidogenic gene mRNA levels after GD12–20 DBP exposure. mRNA levels were quantified using Taqman-based quantitative RTPCR. Group sizes were six control and five DBP-treated litters. All data are the mean ± SD, and an asterisk above a graph indicates a p value < 0.05 compared with control.

were not affected by either DBP dose level. *Srebf2* mRNA levels were not altered by DBP100 exposure but were significantly reduced at the DBP500 dose level. INSIG1 regulates the proteolytic activation of SREBP (Goldstein *et al.*, 2006), and the *Insig1* gene is a transcriptional target of SREBP (Horton *et al.*, 2003). *Insig1* expression was reduced by both DBP100 and DBP500 exposure. Two cholesterol biosynthesis pathway genes (*Idi1* and *Hmgcr*) were examined, with *Idi1* levels reduced by only DBP500 and *Hmgcr* levels reduced by both DBP100 and DBP500. Overall, the expression pattern of steroidogenic genes appeared to correlate with *Srebf2* and cholesterologenic gene expression.

By Western analysis, SREBP2 was quantified in total testis protein extracts. SREBP1 expression was not observed in total testis extracts using two commercial antibodies (NB100-2215R, Novus Biologicals and clone 2A4, Thermo Fisher Scientific) (data not shown). The SREBP2 antibody detected a single band of approximately 165 kDa representing the full-length form of SREBP2 (Fig. 5A). Although the antibody epitope is present in the cleaved form of SREBP2, this form was not observed in the total testis extract. Following DBP100 or DBP500 exposure, no significant change in the total testis level of full-length SREBP2 was observed (Figs. 5B and 5C).

To determine which fetal testis cell types express SREBP, we immunolocalized SREBP in histological sections of GD20 rat testis. SREBP immunostaining was colocalized with ACTA2 (smooth muscle alpha actin), which outlines seminiferous cords and vasculature, and with CYP11A1, a Leydig cell marker. Using the two SREBP1 antibodies, no specific signal associated with any fetal testis cell type was observed (data not shown). Using normal rabbit IgG as the primary antibody resulted in background levels throughout the testis, although a higher nonspecific signal was associated with cells contained within the vasculature (Fig. 6A). SREBP2 immunostaining was associated with all testis cell types, but SREBP2 expression was particularly high in clusters of interstitial cells (Fig. 6B). SREBP2 immunostaining appeared to be largely cytoplasmic. To determine if the abundant SREBP2 interstitial cell staining represented Leydig cell expression, we colocalized SREBP2 and CYP11A1 immunostaining patterns. Serial sections were analyzed because both SREBP2 and CYP11A1 antibodies were generated in rabbit. This analysis revealed a close spatial pattern between interstitial SREBP2 and CYP11A1 immunostaining (Fig. 6C). We concluded that SREBP2 was produced in all fetal testis cell types but displayed highest expression in Leydig cells.

Following both DBP100 and DBP500 exposures, a qualitative difference in SREBP2 immunostaining was observed (Fig. 7A). In control testis, SREBP2 Leydig cell immunostaining was present in numerous, small, and irregularly shaped cell clusters. DBP100 exposure appeared to increase the size of SREBP2-positive Leydig cell clusters, and DBP500 exposure greatly increased the size and reduced the numbers of these clusters. Because DBP exposure is known to cause aggregation of Leydig cells into large interstitial islands (Mahood *et al.*, 2005; Mylchreest *et al.*, 2002), these data provided additional

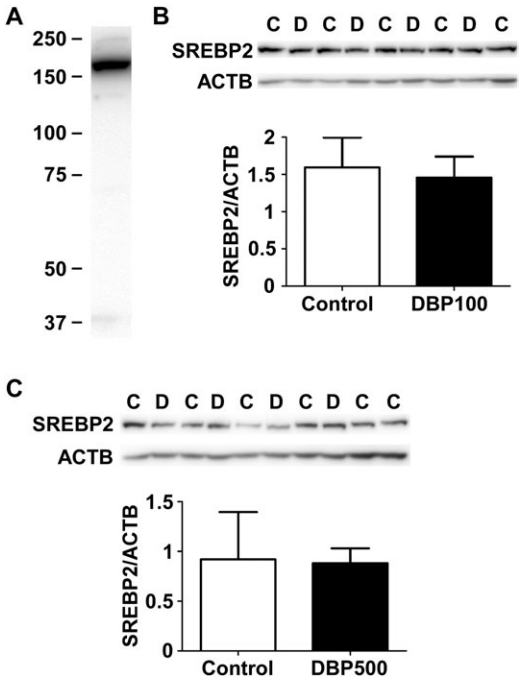

FIG. 5. SREBP2 protein levels in GD20 total testis after GD12–20 DBP exposure. (A) A band of approximately 165 kDa representing full-length SREBP2 was observed. Using normal rabbit IgG as primary antibody, no bands were observed (data not shown). (B) SREBP2 levels following DBP100 exposure. SREBP2 and ACTB proteins were quantified in five control (C) testis samples and four DBP100-treated (D) testis samples. The SREBP2/ACTB ratio is shown in the graph. (C) SREBP2 levels following DBP500 exposure. SREBP2 and ACTB proteins were quantified in six control (C) testis samples and four DBP500-treated (D) testis samples. The SREBP2/ACTB ratio is shown in the graph. Data in the graph are the mean ± SD.

evidence for the localization of highest SREBP2 immunoexpression within Leydig cells.

To measure an effect of DBP exposure on SREBP2 immunoexpression in Leydig cell and seminiferous cord compartments, we used image analysis software to quantify SREBP2 immunostaining in fetal testes after exposure to DBP100 and DBP500. DBP100 did not significantly reduce SREBP2 immunoexpression in entire testis sections, seminiferous cords, or Leydig cells (Fig. 7B). Similarly, DBP500 exposure was without significant effect on SREBP2 immunostaining of the entire testis section or seminiferous cords; however, DBP500 significantly reduced SREBP2 immunoexpression in Leydig cells (Fig. 7C). We concluded that DBP500, but not DBP100, exposure specifically reduced fetal testis SREBP2 levels in Leydig cells.

DISCUSSION

Our data show that phthalate-induced fetal testis endocrine disruption is closely associated with reductions in Leydig cell SREBP2-dependent lipid metabolism gene expression. In the rat, phthalate exposure lowers fetal testis expression of genes in several metabolic pathways. In contrast, mouse phthalate exposure reduces gene expression in only a subset of metabolic pathways, and lipid metabolism gene expression is induced. A recent publication analyzing fetal testis expression microarray data after rat phthalate exposure arrived at similar conclusions to ours concerning the inhibition of several metabolic pathways (Ovacik *et al.*, 2010), but our pathway data extend these observations by comparing the rat data to a high dose-level DBP exposure in the mouse. The resistance to phthalate-induced endocrine disruption observed in the mouse is not because of a lack of molecular effects in the fetal testis. Significant expression changes in several hundred genes were observed in mouse testis after both acute and multiple day DBP exposure. Pathway analysis showed that mouse phthalate exposure reduces gene expression within many metabolic pathways including amino acid, energy, carbohydrate, and certain lipid pathways, but, significantly, inhibition does not extend to the steroidogenic or cholesterologenic metabolic branches. In fact, multiple day DBP250 exposure in the mouse increased fetal testis expression of steroidogenic and cholesterologenic genes. Thus, DBP exposure appears to reduce the activity of several metabolic pathways (carbohydrate, amino acid, and energy) in both mouse and rat fetal testis, but lipid metabolism pathways are increased in the mouse and decreased in the rat.

The mechanism behind the differential rat and mouse phenotypes is unknown, but we hypothesize a species-specific affect of DBP exposure on fetal Leydig cell SREBP2 activity. Endocrine disruption is correlated with both reduced Leydig cell SREBP2 immunoexpression and reduced expression of lipid metabolism genes regulated by SREBP2 transcriptional activity. Furthermore, decreased fetal testis testosterone levels and SREBP2-dependent gene expression have similar time dependence, dose-response, and species dependence. SREBP2 is widely recognized as being the major transcription factor regulating genes controlling cholesterol biosynthesis, and this pathway displayed reduced expression in rat fetal testis as early as 3 h after DBP500 exposure. This timing approximates that of steroidogenic gene expression inhibition, which is likely a major driver of reduced fetal testis testosterone production. Likewise, cholesterologenic and steroidogenic gene expression displayed similar dose-responses after GD12–20 dosing, although expression of some cholesterologenic genes (*Hmgcr* and *Insig1*) was inhibited modestly at DBP100, whereas no significant changes in steroidogenic mRNA amounts were observed at this dose level. Finally, the congruency between effects of DBP on steroidogenesis and cholesterologenesis was supported by similar dose-dependent reductions in rat fetal testis testosterone and total cholesterol levels.

Compared with steroidogenic gene expression and testosterone levels, rat DBP exposure did not reduce expression of cholesterologenic genes, SREBP2 protein, or total cholesterol to the same extent in total testis preparations. For example, testosterone levels after DBP500 exposure were reduced by

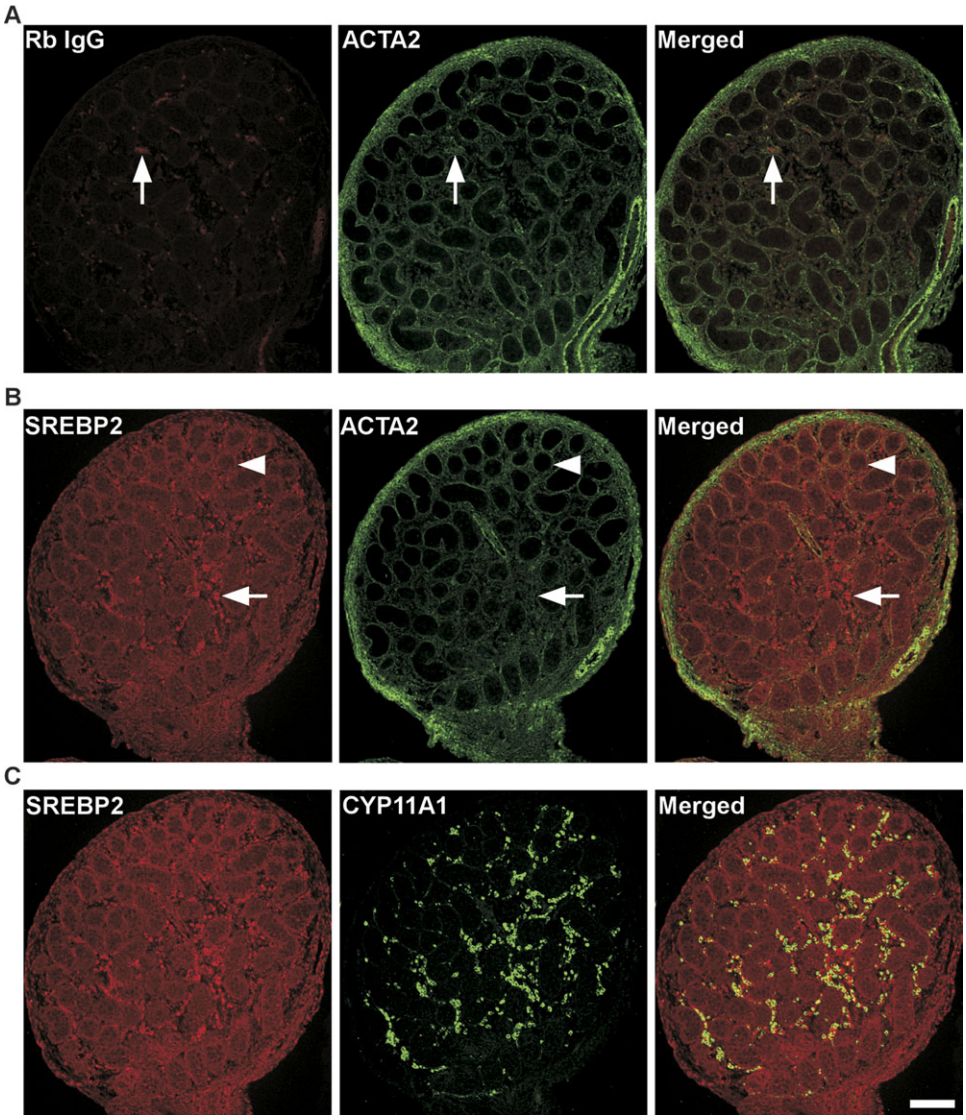

FIG. 6. SREBP2 immunolocalization in control GD20 rat testis. (A) Normal Rb IgG in place of the SREBP2 primary antibody nonspecifically detected cells (arrow) located within the testicular vasculature. ACTA2 immunostaining outlines seminiferous cords and vasculature. (B) SREBP2 immunostaining was present in all testicular cell types but was brightest in clusters of interstitial cells (arrow). Cells in the seminiferous cords also showed relatively high SREBP2 immunoexpression (arrowhead). (C) Using serial sections, bright interstitial cell SREBP2 immunoexpression colocalized to areas of CYP11A1-positive Leydig cells. Bar = 150 μm.

90%, but total cholesterol was reduced only 20%. We speculate that this result stems from DBP profoundly targeting lipid metabolism in Leydig cells but having modest, if any, effects on lipid metabolism in other testicular cell types. This conclusion is supported by data showing that DBP primarily reduces rat fetal testis lipid metabolism gene expression in the interstitial compartment while having little effect on these genes in seminiferous cords (Plummer *et al.*, 2007). Unlike testosterone production and steroidogenic gene expression, which are Leydig cell-specific functions, nearly all mammalian cells have the capacity to generate cholesterol *de novo*, and we observed SREBP2 expression in all testis cell types. Therefore, using whole testis as starting material to examine DBP-induced inhibition of lipid metabolism occurring mainly in Leydig cells would dilute the response. This may be the reason for failing to detect significant reductions in total testis SREBP2 via Western blotting but observing a significant decrease in Leydig cell SREBP2 immunoexpression using a technique that targeted quantification to Leydig cells.

In previous publications, others have suggested a potential role of SREBP inhibition in phthalate-induced endocrine disruption (Lehmann *et al.*, 2004; Shultz *et al.*, 2001). Lehmann *et al.* (2004) examined fetal rat testis *Srebf1* mRNA levels after DBP500 exposure, and, like our results, *Srebf1* mRNA was not reduced. To our knowledge, the studies presented here are the first to examine the effects of phthalate

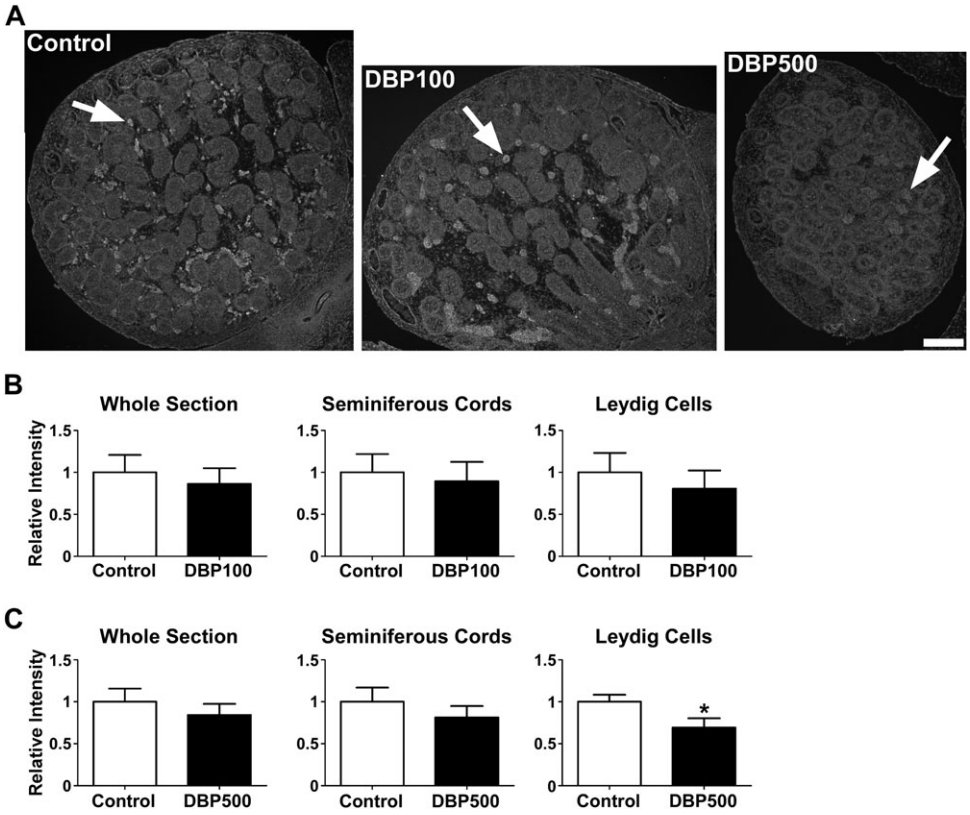

FIG. 7. Qualitative and quantitative changes in GD20 rat testis SREBP2 immunolocalization after GD12–20 DBP exposure. (A) SREBP2 immunoexpression in control, DBP100-treated, and DBP500-treated testes. Within increasing DBP dose levels, SREBP2-positive Leydig cell clusters (arrow) became increasingly larger, and SREBP2 immunostaining signal appeared diminished following DBP500 exposure. Bar = 150 μm. (B) Quantification of SREBP2 immunoexpression following DBP100 exposure. In the three compartments analyzed, no significant effect of DBP100 exposure was observed. Data were quantified in sections from six control litters and three DBP100-treated litters. (C) Quantification of SREBP2 immunoexpression following DBP500 exposure. No significant effect was observed in whole testis sections or the seminiferous cord compartment, but Leydig cell immunoexpression was significantly reduced. Data were quantified in sections from six control litters and five DBP500-treated litters. Graph data are the mean ± SD, and an asterisk above a graph indicates a p value < 0.05 compared with control.

exposure on SREBP2 expression. Androgen receptor antagonist exposure of prostate cell lines can reduce SREBP activation (Heemers *et al*., 2006), and an adult mouse model with elevated testosterone levels shows increased testicular expression of cholesterologenic genes (Eacker *et al*., 2008). These data suggest that phthalates may reduce fetal testis SREBP activity secondary to reduced testosterone production (Shultz *et al*., 2001). Arguing against this hypothesis are data indicating that the androgen receptor may not be expressed in fetal Leydig cells (Majdic *et al*., 1995; but for a dissenting conclusion, see Mylch reest *et al*., 2002) and data showing very few changes in expression of fetal rat testis cholesterol synthesis genes occur following *in utero* exposure to the androgen receptor antagonist flutamide (Mu *et al*., 2006). Using a fetal rat testis culture model, we have observed no effect of androgen or flutamide exposure on expression of cholesterologenic genes (Kamin Johnson, unpublished observations). Furthermore, cholesterologenic and steroidogenic gene changes after phthalate exposure share similar kinetics, suggesting changes in one pathway do not lead to changes in the other. We conclude from these data that reduced androgen receptor activity after phthalate exposure does not play a major role in inhibition of the fetal Leydig cell cholesterologenic pathway.

In the rat model, inhibition of testicular cholesterologenic gene expression occurred despite a reduction in cholesterol levels. At first glance, these observations imply a dysregulation in the testis of the negative feedback loop modulating SREBP activity and cholesterol levels. However, the SREBP-controlling negative feedback loop is sensitive to sterol concentration within the endoplasmic reticulum membrane (Goldstein *et al*., 2006; Radhakrishnan *et al*., 2008), whereas cholesterol can be stored in lipid droplets separated from the endoplasmic reticulum membrane. Leydig cells contain abundant lipid droplets for cholesterol storage, and the diminution of Leydig cell lipid droplets after phthalate exposure (Barlow *et al*., 2003; Lehmann *et al*., 2004) likely accounts for the lowered testis cholesterol content. To account for the simultaneous reduction in both testicular cholesterol content and SREBP-dependent cholesterologenic gene expression, we hypothesize that phthalate exposure decreases storage

of cholesterol in lipid droplets while concomitantly increasing the endoplasmic reticulum cholesterol content. Because oxysterol derivatives of cholesterol also negatively regulate SREBP processing and gene expression activity (Gale *et al.*, 2009; Radhakrishnan *et al.*, 2007), another potential mechanism operating is a phthalate-induced increase in oxysterol production.

Because SREBP2 is a transcription factor and reduced steroidogenic gene expression appears to contribute to phthalate-induced testosterone reductions, the question arises: does SREBP2 modulate steroidogenic gene expression in fetal Leydig cells? Although no data are available in this cell type, SREBP can increase steroidogenic gene expression in other cell types. Mammalian *Star* and *Cyp17a1* promoters contain consensus SREBP binding sites. In nonsteroidogenic human cell lines, both SREBP1 and SREBP2 can bind to the *Star* promoter and increase expression of transfected *Star* promoter/reporter constructs (Christenson *et al.*, 2001; Shea-Eaton *et al.*, 2001). In a human adrenocortical cell line, mutation of the putative SREBP binding site in the *Cyp17a1* promoter and knockdown of SREBP1 both decrease transcriptional activity of a transfected *Cyp17a1* promoter (Ozbay *et al.*, 2006). Thus, the potential exists for SREBP transcriptional activity to increase steroidogenic gene expression in fetal Leydig cells. Defining how SREBP2 is regulated and its functional activity in fetal Leydig cells may provide clues about the phthalate molecular target as well as the signaling pathways governing fetal Leydig cell steroidogenesis.

Although some epidemiological studies suggest that phthalates may perturb human Leydig cell hormone production (Main *et al.*, 2006; Swan, 2008), no direct evidence of human fetal testis endocrine disruption by phthalates is available. Because the experiments presented here did not test human tissue, these data do not speak directly to the endocrine disruption susceptibility of human fetal testes. However, our results could be used to help define the sensitivity of human fetal testes to phthalate endocrine. The question of human sensitivity is highlighted by the differential endocrine disruption observed between mice and rats (Gaido *et al.*, 2007). Unfortunately, the signaling processes controlling rodent fetal Leydig cell hormone production during the critical window of reproductive tract programming are not known (Scott *et al.*, 2009), which impedes understanding of the phthalate endocrine disruption mechanism. We show that endocrine disruption is associated with reduced gene expression of SREBP2-dependent pathways and hypothesize that this may contribute to reduced steroidogenesis. Should an experimental system recapitulating rat sensitivity and amenable to using human tissue become available, SREBP2-dependent gene expression analysis could help determine susceptibility to endocrine disruption.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

National Institutes of Health (P20RR020173 to K.J.J.).

ACKNOWLEDGMENTS

The authors would like to thank Duncan Wallace and Linda Pluta of The Hamner Institutes for Health Sciences for their expert technical help with the DBP50 study and Dr Kevin Gaido (U.S. Food and Drug Administration) for providing several Affymetrix microarray CEL data files.

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